KINETICS AND EQUILIBRIUM OF THE REACTION OF ELLMAN'S REAGENT WITH DOMESTIC HORSE (*Equus caballus*) HAEMOGLOBIN

BY

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B. Tech., M. Tech. (FUTA)

APRIL, 2014.

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A THESIS SUBMITTED TO THE

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D) IN BIOCHEMISTRY

APRIL, 2014.

CERTIFICATION

This is to certify that Omolola Elizabeth OMOTOSHO (Matric. No: CUGP070201) carried out this research work in partial fulfilment of the requirements for the award of Doctor of Philosophy (Ph.D) degree in Biochemistry of Covenant University, Ota, under our supervision.

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DECLARATION

It is hereby declared that this research work titled "Kinetics and Equilibrium of the Reaction of Ellman's Reagent with Domestic Horse (*Equus caballus*) Haemoglobin" was undertaken by Omolola Elizabeth OMOTOSHO.

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DEDICATION

To God alone be all the glory.

MY GLORY AND THE LIFTER UP OF MY HEAD.

This thesis is dedicated to God, my FATHER. All the glory and praise to Him for Whom nothing is impossible, the Omnipotent. Amen. Jesus, I love You. Holy Spirit, my Helper, thank YOU so much. I acknowledge You as my source of wisdom. Thank you for the breakthrough, strength and favour I receive daily and for the grace to complete this work.

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Omotosho, Omolola Elizabeth

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LIST OF ABBREVIATIONS

α	Alpha subunit of haemoglobin
β	Beta subunit of haemoglobin
γ	Gamma subunit of haemoglobin
ATP	Adenosine triphosphate
2,3-BPG	2,3 – Biphosphoglycerate
2-DTP	2,2-dithiobispyridine
Inositol-P_5	Inositol-pentakisphosphate
Inositol-P_6	Inositol hexakisphosphate
Mb	Myoglobin
Hb	Haemoglobin
DTNB	Ellman's reagent: 5,5'-dithiobis (2-nitrobenzoate)
pMB	p-hydroxymercuri(II)benzoate
P_{50}	Pressure at which haemoglobin is half saturated with O_2
PO_2	Partial pressure of oxygen
AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
HbO_2	Oxyhaemoglobin
HbCO	Carbonmonoxyhaemoglobin
metHb	Aquomethaemoglobin
RBC	Red blood cells or erythrocytes
MWC	Monod, Wyman and Changeux
Y	Fractional Saturation
TNB-	Chromophoric product of DTNB reaction; 5-thio (2-nitrobenzoate)
TNBH	Protonated form of TNB^-, the chromophoric product of the DTNB reaction
k_{obs}	Values of the observed rate constant

ABSTRACT

CysF9[93]β exists in two tertiary conformations, ***r*** and ***t***, which are in dynamic equilibrium. The reactivity of the CysF9[93]β sulphydryl group and the oxygen affinity of haemoglobin (Hb) are affected by protons (H^+) and organic phosphates such as inositol hexakisphosphate (inositol-P_6). This study was aimed at determining the effects of inositol-P_6 and pH on the relative populations of the two conformations. The major and minor haemoglobins in horse haemolysate were separated using a column of Whatman's carboxymethylcellulose (CMC 52). Equilibrium studies of the reaction of CysF9[93]β with Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoate) (DTNB), equilibrated for six hours in the presence and absence of inositol-P_6 were carried out at 25°C. The absorbance of the product of DTNB reaction, 5-thio-2-nitrobenzoate, at different concentrations of DTNB and 50 μmol (haem) dm^{-3} was read at 412 nm. The absorbance was then substituted into an appropriate equation derived for the determination of the equilibrium constant, K_{equ}, for the reaction. These experiments were carried out on the oxy, carbonmonoxy and aquomet derivatives of each haemoglobin in the pH range of 5.6 to 9.0. The number of sulphydryl groups in haemoglobin was determined by titrations with p-hydroxymercury(II)benzoate (pMB) and DTNB. The effects of these relative populations on the equilibrium and the kinetics of the reaction of CysF9[93]β of horse haemoglobin with Ellman's reagent were determined. The pseudo-first order kinetics, with the [DTNB] in excess of the Hb concentration, were studied in the presence and absence of inositol-P_6. Values of the observed rate constant, k_{obs}, were plotted against [DTNB] to obtain the apparent second order forward rate constant, k_F. K_{equ} decreased the orders of magnitude between pH 5.6 and 9.0 in the absence and presence of inositol-P_6. Inositol-P_6 increased the affinity of the major and minor Hb for DTNB but decreased the affinity of the minor oxy- and aquomet- Hb. Theoretical calculations from the pH dependence of K_{equ} showed that the pK_a values of the ionisable groups coupled to the DTNB reaction vary between 5.0 and 8.9. The equilibrium constants, K_{rt}, for the ***r*** $\rightleftharpoons$ ***t*** tertiary structure transition, were 0.143 ± 0.05 and 0.446 ± 0.22 for the major and minor stripped horse haemoglobins respectively. In the presence of inositol-P_6, K_{rt} for the major and minor were 2.219 ± 0.79 and 2.214 ± 0.83 respectively. Theoretical calculations from the pH dependence of k_F showed that the pK_a values of the ionisable groups coupled to the DTNB reaction vary between 5.0 and 8.9. The plot of k_{obs} against [DTNB] was linear at each pH, with a non-negligible positive intercept. This is an indication that the reaction of CysF9[93]β of horse haemoglobins with DTNB is reversible. In the presence of inositol-P_6, values of k_F increased across the pH range. Under the same experimental conditions, the binding of inositol-P_6 to horse haemoglobin shifted the tertiary conformation in favour of the ***t*** state; the minor Hb has a higher affinity for DTNB than the major Hb except for aquomet with inositol-P_6.

Keywords: Haemoglobin, *Equus caballus*, Ellman's reagent, kinetics, equilibrium